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Diagnostic Method

This invention relates to a diagnostic method, to a nucleotide sequence comprising a Wilms' tumour suppressor gene (WT1) antisense regulatory region, and to a method of disease detection and prognosis based on the methylation state of the regulatory region.

Wilms' tumour (WT) is a childhood embryonal kidney tumour arising from the malignant transformation of renal stem cells. WT occurs in about 1 in 10,000 children, making it one of the commonest solid childhood tumours.

The human WT1 gene resides on chromosome 11p13 (Call *et al*, (1990) *Cell* **60**, p 509-520; Gessler *et al*, (1990) *Nature* **343**, p774-778; Call *et al*, (1994), US5,350,840) and is genomically organised as 10 exons spanning a 60 kilobase chromosomal region. Intragenic deletions and mutations of the tumour suppressor gene, WT1, have been detected in approximately 10% of Wilms' tumours.

During nephrogenesis i.e. kidney development, WT1 gene expression is controlled in a highly specific manner, increasing as metanephric mesenchymal cells progress towards immature epithelial cells, and attenuating as the cells become more phenotypically mature. The inverse correlation between WT1 expression and the differentiation status of human leukaemic cells along with evidence of expression in ovary and testis and the spinal chord and brain strongly suggest that the function of the WT1 gene product may be pivotal in growth and/or differentiation in a variety of cell types. The WT1 protein, which includes four zinc fingers, is expressed as four isoforms arising from two alternative splice sites (I and II) in the gene. Splice II occurs within the zinc finger domain, inserting or omitting three amino-acids (KTS) between zinc fingers 3 and 4. The WT1 protein without KTS amino acids (WT1-KTS) specifically binds to the EGR site consensus sequence (5'-GCGGGGGCG-3') whereas the WT1 protein with KTS (WT1+KTS) does not. By binding to the early growth response gene (EGR) type site(s) in the promoter regions of genes such as insulin-like growth factor type II (IGF-II), platelet derived growth factor A (PDGF-A), colony stimulating factor-1 (CSF-1), and epidermal growth factor receptor

(EGF-R) WT1 acts as a transcriptional repressor (reviewed in Hastie (1994) *Ann. Rev. Genet* **28,** 523-558, and Menke *et al* (1998) *Int. Rev. Cytol.* **181,** 151-212).

The human WT1 promoter region has been characterised and found to belong to the family of TATA-less, CCAAT-less, GC-rich promoters with multiple responsive sites for the transcription factor Sp1. EGR/WT1 consensus sequences were also identified upstream and downstream of the major transcriptional start site (Hofmann *et al.,* (1993) *Oncogene* **8,** 3123-3132) and the suggestion that these sites may allow WT1 autorepression was subsequently verified using transient transfection assays with the human promoter (Malik *et al., (1994) FEBS Letters 349, 75-78)*

WT1 function is crucial in the normal development of the urogenital system, as demonstrated in WAGR (Wilms tumour, Aniridia, Genitourinary abnormalities and mental Retardation) syndrome and in Denys-Drash syndrome (DDS), diseases characterised by renal and genital abnormalities together with a predisposition to Wilms' tumour (reviewed in Coppes *et al,* (1993) *FASEB J.* **7,** 886-895.)

The evidence for the involvement of WT1 in non-renal tissue differentiation is accumulating. A role in haematopoiesis is suggested by the downregulation of WT1 expression during chemically induced differentiation of HL60 cells (Sekiya *et al, (*1994) *Blood* **83,** 1876-1882) and K562 cells (Phelan *et al, (*1994) *Cell Growth Differ.* **5,** 677-686) Elevated WT1 expression in leukaemic cells relative to normal haematopoeitic progenitor cells (Inoue *et al,* (1997) *Blood* **89,** 1405-1412) and the detection of WT1 mutations in leukaemias (King-Underwood *et al, (*1996) *Blood* **87,** 2171-2179; King-Underwood and Pritchard-Jones, (1998) *Blood 91, 2961-2968*) strongly implicate the involvement of the WT1 gene in leukaemogenesis. Altered WT1 expression has also been shown in breast cancers (Silberstein *et al, (*1997) *Proc. Natl. Acad. Sci. USA 94, 8132-8137*)

Furthermore, antisense WT1 mRNA transcripts with no apparent open reading frames have been detected in foetal kidney and WTs, suggesting a regulatory role for these mRNAs (Campbell *et al, (*1994) *Oncogene* **9,** 583-595; Eccles *et al, (*1994) *Oncogene* **9,**

2059-2063). One such function of these mRNAs may be the formation of RNA heteroduplexes with sense WT1 mRNA, thereby modulating the finite levels of cellular WT1 protein. Previously the inventors reported the identification of an antisense WT1 promoter located in intron 1 which is activated by WT1. This effect of WT1 is reciprocal to that observed on the WT1 promoter, suggesting that the antisense promoter activity is involved in WT1 gene regulation (Malik *et al,* (1995) *Oncogene* **11,** 1589-1595). In addition, it has been demonstrated that expression of ectopic exon 1 RNA can affect the cellular levels of WT1 in an *in vitro* system (Malik *et al,* (1995) *Oncogene* **11,** 1589-1595; Moorwood *et al,* (1998) *J. Pathol* **185,** 352-359) , supporting a regulatory role for antisense WT1 RNAs.

The WT1 antisense transcript may upregulate the levels of WT1 protein (Moorwood *et al,* (1998) *J. Pathol* **185,** 352-359), and aberrations of the control mechanisms for antisense RNA transcription may result in inappropriate temporal and spatial expression of WT1 protein, in turn contributing to tumourigenesis. In this regard, it is interesting to note that WT1 can increase the tumour growth rate of adenovirus-transformed baby rat kidney cells (Menke *et al, (*1996) *Oncogene* **12,** 537-546). The association between epigenetic modification of WT1 antisense regulatory regions, WT1 overexpression and renal tumourigenesis remains unclear, but preliminary studies have indicated that there is a correlation between hypermethylation of WT1 antisense regulatory regions and low WT1 protein, and the converse for hypomethylation. Interestingly, the WT1 antisense promoter locus was identified as a hypermethylated sequence in human breast cancers (Huang *et al,* (1996) *Cancer Res.* **57,** 1030-1034) and breast cancers have been shown to have decreased expression of WT1 (Silberstein *et al, (*1997) *Proc. Natl. Acad. Sci. USA 94, 8132-8137*).

The inventors have identified an antisense regulatory region (ARR) of the WT1 antisense promoter, and have demonstrated that the ARR is part of a differentially methylated region. The WT1 ARR characterised and utilized as the basis of the invention is structurally and functionally distinct from previously described WT1 gene sequences (for example Call *et al,* (1994), US patent 5,350,840). In addition, the inventors have found a correlation between the levels of ARR methylation, and the pathological state of human cells,

Specifically, a variety of cancer cells are shown to differ from their normal counterparts on the basis of epigenetic changes.

Accordingly, a first aspect of the invention provides a nucleotide sequence encoding a WT1 antisense regulatory region comprising at least a portion of, or consisting of, the sequence shown in SEQ1, or at least a portion of a variant, due to base substitutions, deletions and/or additions, of the sequence shown in SEQ.1.

A second aspect of the invention provides a nucleotide sequence encoding a WT1 antisense regulatory region comprising or consisting of the sequence shown in SEQ2, or at least a portion of a variant, due to base substitutions, deletions and/or additions, of the sequence shown in SEQ.2. The WTI antisense regulatory region may be limited to the portion of sequence shown in bold in SEQ. 2, or variants of such a sequence due to base substitutions, deletions and/or additions.

A third aspect of the invention provides a nucleotide sequence encoding a WT1 antisense regulatory region negative regulatory element (NRE) comprising at least a portion of the sequence shown in SEQ.1 or at least a portion of a variant, due to base substitutions, deletions, and/or additions, of the sequence shown in SEQ.1. The nucleotide sequence shown in SEQ.1 may contain several *WT1* antisense regulatory region negative regulatory elements.

Preferably, a nucleotide sequence according to the first, second or third aspects of the invention is a DNA or RNA sequence. Portions of any sequences are preferably functional i.e. they have a biological function of a corresponding native sequence.

A fourth aspect of the invention provides a method of disease detection, diagnosis or prognosis in a subject with cancer, using the differentially methylated state of specific nucleotide sequences, such as the nucleotide sequences in the WT1 ARR region. Genomic epigenetic changes are often regional, for example affecting a variety of gene loci on chromosome 11p15 (Feinberg (1999) Cancer Research (suppl.) **59**, p 1743-1746). The inventors' identification of the chromosome 11p13 region as a target for epigenetic changes

by methylation therefore suggest that other DNA probes/DNA sequences from the 11p13 region, including those derived from the 11p13 genes reticulocalbin and PAX6, may also be utilized for detection purposes in methods according to the invention.

The specific nucleotide sequence(s) may be one or more regulatory elements preferably one or more negative regulatory elements (NRE), for example, one or more NREs within the ARR. The NRE sequence or sequences may be part of the WTI gene, or part of the chromosome 11p13 region, such that a method of disease diagnosis and prognosis in a subject diagnosed with cancer, comprises determining the methylation state of a NRE, or an ARR, of the WT1 gene or chromosome 11p13 region DNA sequence in the subject, and correlating the methylation state of the NRE with the diagnosis and expected long-term recovery prognosis of the subject. For example, in the case of acute myeloid leukaemias (AMLs), hypermethylation of the NRE indicates that the subject has a positive long term recovery prognosis, and hypomethylation of the NRE indicates that the subject is predisposed to relapsing after treatment. In the case of Wilms tumours, hypermethylation of the NRE indicates that the subject has a positive long term recovery prognosis, and hypomethylation of the NRE indicates that the subject is predisposed to relapsing after treatment. In Wilms' tumours, hypomethylation is detected specifically in tumours, and in colorectal cancer cell lines, hypomethylation correlates with tumourigenic potential. However, in other cancers, hypermethylation of the specific nucleotide sequence or sequences may indicate the presence of cancer cells and/or a predisposition of the subject to relapsing after treatment, whereas hypomethylation of the specific nucleotide sequence or sequences may indicate the absence of cancer cells and/or that the subject has a positive long term recovery prognosis For example, see figure 1(e). The diagnostic application is underlined by the hypomethylation in WTs, as opposed to the hypermethylation of other renal tumours, such as primitive neuroectodermal tumour (PNET) and clear cell sarcoma of the kidney (CCSK) (see figure 1D).

The methylation state may be determined by restriction of the WT1 antisense regulatory region using enzymes such as *Bsh*1236I, *Spe*I and *Kpn*1 in combination. Bsh1236I is an isoschizomer of Bst UI. Bsh1236I cuts at the restriction sequence CGCG only when there is no CpG methylation. Methylated sequences are not restricted by *Bsh*1236I. Therefore,

the restriction pattern obtained for a nucleotide sequence which has been restricted with Bsh1236I gives a different band pattern depending on whether the Bsh1236I sites in the nucleotide sequence are methylated or not. Other commercially available enzymes may also be used, with one or more being able to distinguish between methylated and unmethylated DNA.

The methylation state may be determined using a PCR-based assay system. Such a PCR-based assay system may involve the use of sodium-metabisulphite. This has the effect of converting all unmethylated cytosine residues to uracil residues. Preferably, the PCR reaction uses the following primers to amplify at least a portion of the WT1 antisense regulatory region:

Tf: 5'-GGGTGGAGAAGAAGGATATATTTAT-3'.
Tr: 5'-TAAATATCAAATTAATTTCTCATCC-3'.

TfN: 5'-GATATATTTATTTATTAGTTTTGGT-3' (nested primer).
TrN: 5'-AAACCCCTATAATTTACCCTCTTC-3' (nested primer).

The conditions used in the PCR reaction are the same as the conditions mentioned later in the specification. The PCR products obtained from the PCR reaction, as described below, may then be cloned and sequenced. The PCR products may be cloned into a vector such as pGEM-T (Promega). Alternatively, the PCR products may be sequenced directly. Once sequenced, any methylated cytosine residues will remain readable as 'C' in the nucleotide sequence, whereas unmethylated cytosines will appear as 'T' residues in the sequence.

The nested PCR reaction involves the following primers.

Tf: 5'-GGGTGGAGAAGAAGGATATATTTAT-3'.
Tr: 5'-TAAATATCAAATTAATTTCTCATCC-3'.

TfN: 5'-GATATATTTATTTATTAGTTTTGGT-3' (nested primer).
TrN: 5'-AAACCCCTATAATTTACCCTCTTC-3' (nested primer).

A fifth aspect of the invention provides a method of cancer detection in cells derived from a subject comprising detection of tumour-specific alteration of genomic imprinting. Any bi-allelic expression of tumour-specific genes may indicate the presence of tumourgenic cell proliferation if the normal tissue expresses the gene monoallelically. Alternatively, with some cancers, the normal tissue may be biallelic, and the cancer monoallelic. Additionally, methylation changes may be accompanied by changes in gene expression through silencing or enhanced gene expression, irrespective of allelic contributions to gene dosage (reviewed in Jones (1996), Cancer Research **56**, p2463-2467)

The tumour-specific alteration of genomic imprinting may be detected by reverse transcription PCR (RT-PCR). This allows relatively fast detection of altered genomic imprinting by visual analysis of the RT-PCR products on an electrophoretic gel.

The method may be used in the detection of WT in a subject, and may detect alteration of genomic imprinting of WT-specific genes such as the WT-1 gene.

The altered genomic imprinting detected may be relaxation of genomic imprinting, loss of imprinting, or gain of imprinting.

The RT-PCR may use two primers designed to anneal to a tumour-specific gene sequence on opposite sides of an allelic polymophism which introduces a restriction-site in one allele only. For example, in the case of WT, the RT-PCR may use the following primers:

Primer 1: WT18 [CTTAGCACTTTCTTCTTGGC]
Primer 2: WITKBF2 [TTGCTCAGTGATTGACCAGG]

A sixth aspect of the invention provides a method of treating a subject with a specific cancer, comprising altering the genomic imprinting of a tumour-specific gene. This may involve relaxation of the genomic imprinting, or reversal of relaxed genomic imprinting.

A seventh aspect of the invention provides a diagnostic kit, assay or monitoring method using a method according to a fifth aspect of the invention.

An eighth aspect of the invention provides a method of detection of the methylation state of a WT1 antisense regulatory region comprising detection of a tumour-specific alteration in genomic imprinting using a method according to a preceding aspect of the invention, and correlating a detected alteration in genomic imprinting with differential methylation of the WT1 antisense regulatory region. For example, relaxation of genomic imprinting may be correlated with hypomethylation of the WT1 antisense regulatory region.

Nucleotide sequences, and methods of disease diagnosis, detection and prognosis in accordance with the invention will now be described, by way of example only, with reference to accompanying Figures 1(A) to 3(B), and SEQ.1 to SEQ. 3 in which;

Figure 1(A) shows the probe used for the detection of methylation for Southern blotting; and

Figure 1(B) shows a Southern blot of three acute myelogenous leukaemia (AML) DNAs and a normal peripheral blood lymphocyte DNA; and

Figure 1(C) shows a Southern blot of DNAs from a non-tumourogenic and a highly-tumourgenic colorectal cell line; and

Figure 1(D) shows a Southern blot of matched normal kidney and WT samples, matched normal kidney and PNET or CCSK DNAs and a foetal kidney control; and

Figure 1(E) shows Southern blot analysis of breast tumour DNAs for changes in the ARR methylation status.

Figure 2 shows the nucleotide sequence of a WT1 ARR, with the primer hybridisation sites indicated by arrows; and

Figure 3(A) is a schematic diagram showing the primers on either side of the antisense WT1 RNA splice used for RT-PCR; and

Figure 3(B) shows a southern blot of the antisense WT1 RNA RT-PCR products; and

SEQ.1 shows a nucleotide sequence of the WT1 ARR; and

SEQ.2 shows a nucleotide sequence of a negative regulatory element of a gene encoding WT-1; and

SEQ.3 shows the nucleotide sequence of a WT1 antisense region (Gessler, M & Bruns (1993) Genomics 17: 499-501) with the RT-PCR primers shown as arrows and the exonic sequences indicated in bold.

1. Cloning and characterisation of WT1 genomic sequences

The WT1 cDNA and WT1 promoter region were cloned from a human foetal kidney cDNA library (Clontech) and a human B-cell genomic library (λSha2001, kindly supplied by T. H. Rabbitts, Medical Research Council, Cambridge) respectively. For each library, Plaque screen filters (Du Pont) were prepared *in situ*from 1 x 10^6 phage (Benton, W. D. and Davis, R. W. (1977). *Science*, **196**, 180-182). Filters were hybridized in 6x SSC (1x SSC = 0.15 M NaCl, 0.015 M sodium citrate), 5x Denhardts solution, 0.5% SDS and 100 μg/ml salmon sperm DNA at 65°C. Washing was performed at high stringency (0.1x SSC, 0.5% SDS, 65°C). For the cDNA library, a partial WT1 cDNA obtained by PCR amplification was used as probe. The DNA sequence of a full-length cDNA isolated from the cDNA library was determined by the dideoxy chain terminator method (Sanger, F., *et al* (1977). *Proc. Natl. Sci. USA*, **74**, 5463-5467), and a 700 bp fragment from the 5' terminus of the cDNA was used for probing the genomic library. Probes were radiolabelled with [α-^{32}P]dCTP (Amersham) according to the random primer method (Feinberg, A. P. and Vogelstein, B. (1983). *Biochem. Biophys. Res. Commun.*, **111**, 47-54).

Genomic clones corresponding to the 5'-end of the WT1 gene were subcloned and characterised by restriction analysis according to standard methodology (Sambrook, J., *et al* (1989). *Molecular Cloning, Vols 1 and 2, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.*). DNA sequences were determined by the dideoxy chain terminator method (Sanger, F., *et al* (1977). *Proc. Natl. Acad. Sci. USA*, **74**, 5463-5467) and by Δtaq cycle-sequencing according to the manufacturers instructions (USB-Amersham). The functional assessment of DNA from intron 1 of the WT1 gene was carried out by transient transfection of reporter gene constructs with various WT1 intronic sequences directing gene expression (Malik, K., *et al* (1995) *Oncogene*, **11**, 1589-1595).

2. Differential Methylation assays

Human genomic DNAs are purified by standard phenol-chloroform extraction procedures (Sambrook, J., *et al* (1989). *Molecular Cloning, Vols 1 and 2, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.*). Based on the DNA sequence of the intronic region (see Figure 2), digestion by restriction enzyme Bsh1236I (MBI Fermentas) has been

selected to examine methylation of the intronic region. This enzyme cuts at the restriction sequence CGCG only when there is no CpG methylation; methylated sequences are not restricted. Our work has established that differential methylation is conveniently detected within a KpnI - SpeI (New England Biolabs) fragment of 850 bp, which contains 4 potential Bsh1236I sites (see Figure 1) . Depending on whether these sites are methylated or unmethylated, a characteristic banding pattern is observed after digestion of genomic DNAs with a combination of KpnI, SpeI, and Bsh1236I, Southern blotting and hybridisation with a radiolabelled DNA probe (Sambrook, J., *et al* (1989). *Molecular Cloning, Vols 1 and 2, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.*) defined by the KpnI and SpeI sites in the intronic sequence (Figures 1 and 2).

Figure 1(D) shows a Southern blot of matched normal kidney and Wilms' tumour samples. All WT samples were confirmed as having no loss of heterozygosity. Also shown are matched normal kidney and PNET or CCSK DNAs.

As shown in Figure 1 (D), the pattern of differential methylation successfully distinguishes between normal kidney DNA and Wilms' tumour DNA (panel A), leukaemic cells from patients with varying prognosis and normal lymphocytes (panel B) and also highly tumourigenic and non-tumourigenic colonic cell-lines (panel C). The results shown in panel C suggest that this change may be associated with the tumourigenic process and may therefore be relevant to cancers other than only Wilms' tumour.

In Wilms' tumours, hypomethylation of specific nucleotide sequences correlates with the tumour state. However, in other cancers, this correlation may be inverted, such that hypermethylation of specific nucleotide sequences corresponds to the methylation status of tumour cells, and hypomethylation may indicate normal cells. An example of this is shown in figure 1(E) , with Southern blot analysis of normal breast tissue DNA and breast tumour DNAs for changes in the ARR methylation status. Four infiltrating ductal carcinomas of varying aggressiveness all showed increased methylation of the WT1 ARR compared to the normal breast tumour DNA. Therefore the relative differential methylation comparing normal tissue and tumour tissue may be utilised diagnostically.

3. PCR-based assay system

Tumour cells and normal cells may be distinguished by their epigenotype as previously outlined. Knowledge of the DNA sequence of the WT1 antisense regulatory region has made it possible to develop a PCR-based assay system to allow the determination of the methylation status of samples, which will require less biological material. This method involves introducing CpG dinucleotides which are not part of a restriction enzyme recognition sequence by treatment of genomic DNA samples with sodium-metabisulphite (Merck), thereby converting all unmethylated cytosine residues to uracil (Paulin, R., *et al* (1998) *Nucleic Acids Research* **8**, 4777-4790). Specific regions of interest in the WT1 intronic sequence can then be amplified using primers specific for both strands of DNA. The PCR bands obtained can be directly sequenced or cloned using a commercially available vector such as pGEM-T (Promega) and analysed by DNA sequencing. Any methylated cytosine residues will remain readable as 'C' in the DNA sequence, whereas unmethylated cytosines will appear as 'T'.

Alternatively, after the first round of PCR on bisulphite-treated DNA, nested primers which include one specific for the methylated Bsh1236I site shown to be commonly differentially methylated (boxed in Figure 2), or one specific for the unmethylated *Bsh*1236I site (i.e. specific for C→T conversion) may be employed, permitting discrimination between methylated and non-methylated sequences by visualisation of the PCR products, i.e. if a primer specific for the methylated *Bsh*1236I site is used, a PCR product will only be observed if the *Bsh*1236I site in the sample is methylated, otherwise, no PCR amplification will occur.

Illustrative primers which may be used for methylation-specific PCR are shown below, and their hybridisation positions to the WT1 sequence are shown by arrows in Figure 2 for top-strand amplification. Allowing for C→T conversion, these are:

Tf: 5'-GGGTGGAGAAGAAGGATATATTTAT-3'.

Tr: 5'-TAAATATCAAATTAATTTCTCATCC-3'.

TfN: 5'-GATATATTTATTTATTAGTTTTGGT-3' (nested primer).

TrN: 5'-AAACCCCTATAATTTACCCTCTTC-3' (nested primer).

Typical primary amplifications are conducted with Amplitaq (Perkin-Elmer) with 100 ng. of bisulphite-treated DNA in buffer supplemented with 3mM $MgCl_2$. Amplification conditions are 3 mins. denaturation at 94°C, followed by 35 cycles of denaturation at 94°C for 30 secs, annealing at 50°C for 30 secs, and extension at 72°C for 90 secs. A final extension of 5 mins at 72°C completes the reaction. Secondary PCR with the nested primers employs the same conditions, but using 1/100th of the primary PCR reaction and 24 cycles.

4. Correlation of the methylation state of the (NRE) with long term disease prognosis

The inventors have detected a correlation between the methylation state of the ARR and the diagnosis and long term disease prognosis in subjects with cancer. The diagnostic potential is shown by the hypomethylation in WTs, as opposed to the hypermethylation of other renal tumours, such as primitive neuroectodermal tumour (PNET) and clear cell sarcoma of the kidney (CCSK) (see figure 1D). AML subjects with hypermethylated ARR, responded well to treatment and made a full recovery. However, subjects who had an unmethylated NRE, and relapsed, were refractory to treatment.

Therefore, the methylation state of the NRE can be used as a potential early indicator of the long term diseased prognosis. Subjects who have an unmethylated NRE can be kept under closer observation for early detection of relapse. This will maximise their chances for recovery. However, the expense of such close observation post-treatment is not necessary with subjects with unmethylated NRE, as these patients are expected to respond well to treatment once any relapse has been detected by normal routine checking.

In pilot studies with AMLs, hypermethylation of specific nucleotide sequences corresponds to a predicted positive long term prognosis of the subject with the AML, and hypomethylation corresponds to a predisposition of the subject to relapsing after treatment. However, in other cancers, this correlation may be inverted, such that hypermethylation of specific nucleotide sequences corresponds with a predisposition to relapsing after treatment, and hypomethylation may indicate a positive long term prognosis for recovery.

Therefore, decisions on the best methods of therapy to suit the subject can be made in the light of an educated expectation of how the subject is expected to respond to treatment in the event of a relapse of their cancer condition.

Therefore, it is the differential methylation that is the determinant in developing long term prognosis for subjects diagnosed with cancer.

5. Genomic imprinting of the WT1 gene

The WT1 allele specific methylation pattern observed in normal kidney cells strongly indicates that there is genomic imprinting of the WT1 ARR/NRE (Antisense Regulatory Region/Negative Regulatory Region) and tumour-specific relaxation of genomic imprinting in Wilms' tumours.

Genomic imprinting is the phenomenon by which maternal or paternal copies of a gene can be selectively expressed, with methylation of DNA serving as the regulatory signal. Loss of such a signal can lead to an altered dosage of gene expression that can be deleterious to normal cell growth. For example, the *IGF2* gene exhibits loss of genomic imprinting control of *IGF2* and is overexpressed in WTs (Feinberg, A. P. (1999) *Cancer Res. (suppl.), 59*: 1743s-1746s). As *IGF2* is a growth factor, this may easily contribute to uncontrolled proliferation associated with tumourigenesis.

In order to determine whether the differential methylation of the *WT1* ARR/NRE is accompanied by allele specific expression of the WT1 antisense RNA (*WT1-AS*), reverse transcription-PCR (RT-PCR) analysis was conducted on foetal and normal kidney cells, and WT cells. Primers either side of the antisense *WT1* RNA splice (see SEQ3 and Figure 3A) (Gessler, M., and Bruns (1993), *Genomics*, *17*: 499-501, 1993) were used for RT-PCR:

Primer 1: WT18 [CTTAGCACTTTCTTCTTGGC]

Primer 2: WITKBF2 [TTGCTCAGTGATTGACCAGG].

Typical reaction conditions used for the RT-PCR were annealing of the reverse primer to 1 μg of total RNA by heating to 60°C for 5 mins, followed by quenching on ice, followed by reverse transcription carried out with Super RT (HT Biotechnologies, Cambridge, U.K.)

reverse transcriptase at 50°C for 60 mins. This was followed by PCR cycling as follows:

95°C, 3 mins. (1 cycle);
94°C, 15 secs., 60°C, 30 secs., 72°C, 60 secs. (2 cycles);
94°C, 15 secs., 58°C, 30 secs., 72°C, 60 secs. (2 cycles);
94°C, 15 secs., 56°C, 30 secs., 72°C, 60 secs. (10 cycles, 20 for antisense product); and
94°C, 15 secs., 56°C, 30 secs., 72°C, 60 secs.with 20 secs. extension per cycle (20 cycles).

The PCR products obtained were digested by adding the restriction enzyme *MnlI* directly to the PCR mix and incubating for 60 minutes at 37°C. The PCR products were then separated on 2% agarose gels and then alkali blotted onto Hybond N^+ membrane and hybridised with a ^{32}P-labelled antisense cDNA probe. The sequence of the probe is shown in bold between WT18 and WITKBP2 in SEQ. 3. The following primers were used as DNA controls:

Primer 1: WITKBF2 [TTGCTCAGTGATTGACCAGG]
Primer 2: WITKBR2 [TTGGCTGGAAAGCTTGCAGC]

The *MnlI* polymorphism (Grubb, G. R. *et al* (1995) *Oncogene*, **10**: 1677-1681) utilised is marked by an asterisk in figure 3A, and results in RT-PCR products of 286 **and** 222bp for biallelic expression, or alternatively major allelic bands of 286bp **or** 222bp for monoallelic expression.

As shown in figure 2B, expression of WT1-AS in normal kidney samples that have one methylated and one unmethylated allele, only occurs from one allele, confirming genomic imprinting. However, WTs display biallelic expression of WT1-AS, thus revealing a relaxation of imprinting control in WTs. The net increase arising from expression of both *WT1-AS* alleles may thus serve as an additional marker of the differential methylation pattern detected in Wilms' tumours.

This altered imprinting is likely to be present in cancers other than WT, and therefore, altered imprinting control of specific genes may provide a marker for the detection or

diagnosis of various cancer types in a patient. Furthermore, as epigenetic modifications of DNA are reversible, detection of altered imprinting control and/or the diagnosis of methylation changes should also facilitate therapeutic strategies based on enzymes such as DNA methyltransferases and demethylases, or by chemical compounds (Jones P.A. and Laird P.W. (1999), *Nature Genetics*, **21**, p163-167). This would enable control of gene expression and permit therapies that are contingent on appropriate gene control.

SEQ.1

CTCGAGGATCCAGAGACGGCCTTGATCCTCTCCCCTGGGGTTTGGCCTTGGCGCTCTGAT

GGCCATTTCCACATTTTTGAGAGTTGATGCCCTTGCCTCTCACAGCCCAAGTCTTGGGCC

AGGCCCTGCATTCCTGGGGAAGCAGCAGGAACCCTGGAAATCCAAAGAATAAACCCAGAA

TCTCGAGGGCCACCCTTGCCCACTCCAGGATAGCAGCCGGAGCGCTTCTCACATCCAAGC

TGCCCAATGAGCCTCAAGGGCTGGGTAAGATGGACCCATCTGTTTTCACTGCAAGACAAA

ACTTAAACCTGGAGATGGTGCTTCCAGGCTATATGACTTGAATCTAGGGCCCTCTCTCCA

TTGGGCTTTTTCTCCAGGGTGGAGAAGAAGGATACATTCACCTACTAGTCCTGGTCCCCT

TTTAACTTTTTCTCCATGGCAGCCACGCCTGTATATTACAGAAGAATCCAGATATTTTCC

AGAAGTGTAATACCTGCTGGCTGCAAAACCCACAGTCCCACCCCCCACGACATGTGATAA

GATCCCAGGCACCAGACCTGCCCTGAAAAGGGCTGGACAAGGGACCCAAACGAAGCGACA

GAACCCAGGTTTCAAAAATCCCCTAGAAGTACTAAAAAGATAATGGCGTAGTAGTATTTT

GTGCCCCAGGGGCATGGATTCGATGGTTTCTCAACCGCCTCCAAATAGCACACATGCAGA

CAGTGCTCTCGGATTCATTGTTTCTCAGTCACAGATGTTTAGATGGGTTGCCGAGTTCCA

TATTTAAAGCCCCAAGAGGGTGGTGGGTAGCGCTTCTGCATCTATGGAGTATAACTTCAA

GCCGGACCCAATCTCCAGGTTGCCCATCTCAGCTGTCCTCTTATAGACGGGGACACTGAG

ACCTAGAAACTCCCCAAAAGTAACACCAGCCTGCTAAACAAAGGTGGCGCGATCTGATCA

AAGAACACAAGCCTCAGCGATTAGTAAGTTGTCCAACGCCCCTTGAGTAGAAACACTAAT

TTACTAACTAAAAGCATAGAGTGGAGGCTTCCCTTGGGTCTGCTTGCGGTTCCTCCACAG

GACAGTGATCCCAGATTCTCCCGAAGAAAAGGGCGGTTTCGATTTCTCCAAGGCTTCGCG

GGGGCCGGGTGCTCCTGGTTAAACTAAGGTAGGAGCGGCCTGAAGACGCGCGTTTAGAAG

GCGCCGGGTGAAGGCGGGCAACAAGGCAGAGCCCTTCTCCCGAGCCTTGGGCGAAGGTAC

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WT18

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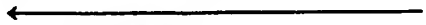

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